



BRAZILIAN JOURNAL OF MICROBIOLOGY

<http://www.bjmicrobiol.com.br/>


Environmental Microbiology

Isolation and identification by 16S rRNA sequence analysis of plant growth-promoting azospirilla from the rhizosphere of wheat



Khadija Ayyaz, Ahmad Zaheer, Ghulam Rasul, Muhammad Sajjad Mirza*

National Institute for Biotechnology and Genetic Engineering (NIBGE), Jhang Road, Faisalabad, Pakistan

ARTICLE INFO

Article history:

Received 23 July 2014

Accepted 24 November 2015

Available online 19 April 2016

Associate Editor: Iêda de Carvalho Mendes

Keywords:

Azospirillum brasilense

Azospirillum zeae

BOX-PCR

Indole-3-acetic acid (IAA) production

Phosphate solubilization

ABSTRACT

The main objective of the present study was to isolate phytohormone-producing, phosphate-solubilizing strains of *Azospirillum* from wheat to be used as inoculants for plant growth promotion. Five *Azospirillum* strains were isolated from the rhizosphere of field-grown wheat (*Triticum aestivum* L.), and it was confirmed by BOX-polymerase chain reaction (PCR) that the isolates were different and not re-isolates of the same strain. Sequence analysis of the PCR-amplified 16S rRNA gene indicated that four isolates showed maximum similarity to *Azospirillum brasilense* and one isolate showed maximum similarity to *Azospirillum zeae*. This is the first report indicating the presence of an *A. zeae* like isolate in the wheat rhizosphere in Pakistan. The bacterial isolates were characterized for their plant growth-promoting traits, phosphate solubilization, and indole-3-acetic acid (IAA) production. None of the isolates showed phosphate solubilization activity in the commonly used Pikovskaya medium. However, all strains (except AzoK4) exhibited ability to solubilize tricalcium phosphate (TCP) in modified Pikovskaya medium in which sucrose was replaced by Na-malate, as well as in TCP-supplemented Luria-Bertani (LB) medium. Organic acids, such as acetic, citric, lactic, malic, and succinic acids, were detected in culture supernatants of the tested *Azospirillum* strains. All strains exhibited ability to produce IAA in the growth medium, except *Azospirillum* sp. AzoK1. Among the strains tested, the maximum IAA production ($30.49 \pm 1.04 \text{ mg L}^{-1}$) and phosphate solubilization ($105.50 \pm 4.93 \text{ mg L}^{-1}$) were shown by a pure culture of *Azospirillum* sp. AzoK2. In pot experiments, single-strain inocula of *Azospirillum* sp. AzoK1 and AzoK2 improved wheat plant growth.

© 2016 Published by Elsevier Editora Ltda. on behalf of Sociedade Brasileira de Microbiologia. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author.

E-mail: sajjadmirza58@gmail.com (M.S. Mirza).

<http://dx.doi.org/10.1016/j.bjm.2015.11.035>

1517-8382/© 2016 Published by Elsevier Editora Ltda. on behalf of Sociedade Brasileira de Microbiologia. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Plant growth-promoting rhizobacteria (PGPR) isolated from the rhizosphere or root surfaces comprise an active component of biofertilizers. Several direct or indirect mechanisms are adopted by these microorganisms to enhance the plant growth, including biological nitrogen fixation, solubilization of insoluble minerals, production of phytohormones, plant relief from stress, and protection against pathogens.¹ Among PGPR, strains of *Azospirillum* are most extensively studied for their beneficial effects on the growth and yield of many agronomically important crops.² It has been reported that inoculation with these diazotrophic bacteria reduces the use of chemical nitrogen fertilizers.³ Indole-3-acetic acid (IAA) production by *Azospirillum* spp. has been proposed to play a major role in root proliferation and consequent plant growth promotion.⁴ IAA is produced by *Azospirillum* during all growth stages, and its synthesis continues well after the stationary phase.⁵ This feature makes the bacteria especially qualified for plant growth promotion when the effect lasts weeks or months after inoculation. The amount of IAA produced varies greatly among different species and is also influenced by culture conditions, the growth stage, and availability of a substrate(s).⁶ It was reported that wheat inoculation with *Azospirillum* strains resulted in a yield increase of 31%.⁷ Inoculation with *Azospirillum* also increases the uptake of mineral nutrients such as N, P, K, and Ca by wheat.⁸

Soils usually contain sufficient reserves of phosphorus to support plant growth if P is in available forms. Unfortunately, phosphorus may be present in water-insoluble forms when it is not readily accessible to plant roots. Therefore, there is a dire necessity to make phosphorus more available to plants.⁹ Phosphorus solubilization activity has been reported for many genera of PGPR, including *Azospirillum*. PGPR produce organic acids, which convert insoluble phosphorus into available forms, i.e., primary and secondary orthophosphates.^{10,11} The organic acids produced by PGPR include malic, acetic, citric, oxalic, lactic, formic, gluconic, and 2-keto-gluconic acids.^{12,13} In pure bacterial cultures, organic acid production has been demonstrated upon utilization of various sugars and sugar alcohols, such as sucrose, glucose, fructose, D-xylose, and mannitol.¹⁰ Phosphate-solubilizing microbes are considered important members of PGPR, and their application as biofertilizers has been shown to improve growth of cereals and other crops.¹³

In the present study, five *Azospirillum* strains were isolated from the rhizosphere of wheat, followed by strain

identification using DNA-based techniques [BOX-polymerase chain reaction (PCR) and analysis of 16S rRNA gene sequences]. After confirmation of IAA production, P solubilization and amplification of the *nifH* gene, as effects of inoculation with selected *Azospirillum* strains was studied on wheat plants.

Materials and methods

Study site

This study on isolation of plant growth-promoting azospirilla continued for 16 months (from January 2012 to April 2013) at the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. The NIBGE is located in a subtropical area of Pakistan (31°23'36.2"N and 73°01'39.9"E) at an elevation of 184 m.

Isolation of *Azospirillum* strains from the rhizosphere of wheat

Isolation of *Azospirillum*-like strains from the rhizosphere of wheat (*Triticum aestivum* L.) was carried out by an enrichment culture technique using semi-solid, N-free malate (NFM) medium.¹⁴ Twenty-five rhizospheric soil samples were collected from field-grown plants at the tillering stage, i.e., 40 days after sowing. Soil suspensions were prepared by adding 1 g of soil to 10 mL of water, and 100 μ L was transferred to 1.5 mL microcentrifuge tubes containing 0.9 mL of semi-solid NFM medium. The inoculated microtubes of NFM were incubated for 24–72 h at 30 °C. The growth of *Azospirillum*-like cells showing typical spiral motility was detected under a light microscope at 1000 $\times$ magnification (Akon, Japan). Six consecutive transfers from inoculated to fresh NFM microtubes were made to obtain an enriched culture. Serial dilutions (10 $\times$) of the enriched culture were made, and 100- μ L aliquots of 10⁻³–10⁻⁵ dilutions were spread on NFM agar plates and incubated for 4–6 days at 30 °C. Bacterial colonies were picked from the plates, transferred to NFM semi-solid medium, and incubated for 24–48 h at 30 °C. Bacterial growth obtained in NFM medium was streaked on Luria-Bertani (LB) agar plates,¹⁵ and the plates were incubated at 30 °C for 24–72 h to obtain single colonies. After repeated streaking and transfer of single bacterial colonies, five pure isolates were obtained. For long-term storage, bacterial growth obtained in LB broth was kept in the medium with 15–20% glycerol at –80 °C.

Table 1 – PCR primers used for amplification of 16S rRNA, *nifH*, and BOX-PCR in this study.

Name of primer	Primer sequence	References
BOXAIR	5'-CTACGGCAAGGCGACGCTGACG-3'	29
PA forward (16S rRNA)	5'-AGACTTTGATCCTGCTCAG-3'	45
PH reverse (16S rRNA)	5'-AAGGAGGTGATCCAGCCGCA-3'	45
PolF (<i>nifH</i>)	5'-TGCGAYCCSAARGCBGACTC-3'	46
PolR (<i>nifH</i>)	5'-ATSGCCATCATYTCRCGGA-3'	46

Differentiation of *Azospirillum* strains by BOX-PCR

All chemicals, except the primers used for PCR, were purchased from Fermentas, Germany. Each PCR reaction mixture (25 μ L) contained 0.15 μ L of 5 U μ L⁻¹ Taq DNA polymerase, 5 μ L of 10 $\times$ Taq polymerase buffer, 5 μ L of 25 mM MgCl₂, 2 μ L of 5 mM deoxynucleotide triphosphates (dNTPs), 1.5 μ L of 10 μ M BOXAIR primer (Table 1), and 25 ng of DNA template extracted by CTAB method.¹⁶ The reaction mixture was incubated in a thermocycler (Eppendorf, Germany) at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 1.5 min, and final extension at 72 °C for 10 min. Amplified PCR products of different sizes were separated on 1% agarose gel electrophoresis and stained with ethidium bromide. Gels were viewed under ultraviolet (UV) light and photographed using a gel documentation system (CN-1000/26MX, Vilber Lourmat, France).

Amplification of 16S rRNA and *nifH* genes

For amplification of the 16S rRNA and *nifH* genes, a reaction mixture of 25 μ L, containing 15 ng of template DNA, 0.12 μ L of 5 U μ L⁻¹ Taq DNA polymerase, 2.5 μ L of 10 $\times$ Taq polymerase buffer, 1 μ L of 5 mM dNTPs, 2 μ L of 25 mM MgCl₂, and 1 μ L of each 10 μ M forward and reverse primers, was prepared. The primer set PH/PA was used for amplification of 16S rDNA, and the primer set PolF/PolR was used for amplification of *nifH* (Table 1). The program used for PCR amplification of 16S rDNA included following steps: initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min, and final extension for 10 min at 72 °C. The same thermal conditions were used for amplification of *nifH*, except that the annealing temperature was 48 °C.

Identification of isolates by 16S rRNA sequence analysis

The PCR products of the 16S rRNA gene were eluted from an agarose gel using the GeneJET™ Extraction Kit (Fermentas, Germany), and the purified PCR products were sequenced commercially by Macrogen, South Korea. The sequence data were assembled and analyzed using the LaserGene sequence analysis software package (DNA Star, Inc., Madison, WI, USA). Sequence similarity searches using the Basic Local Alignment Search Tool (BLAST) were performed by comparing the sequences obtained to other microbial sequences in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/BLAST/>). For construction of a phylogenetic tree, the 16S rRNA gene sequences of type strains and other strains closely related to our five isolates were obtained from the NCBI database. Multiple sequence alignments were performed by the ClustalX program.¹⁷ Phylogenetic trees were constructed by the maximum-likelihood method based on the Jukes–Cantor model¹⁸ using MEGA version 6.¹⁹ All positions containing gaps and missing data were eliminated. There were a total of 816-base pair (bp) positions in the final dataset.

Quantification of IAA produced by *Azospirillum* strains

For detection and quantification of IAA produced by the bacterial isolates, cultures were grown in Erlenmeyer flasks containing 40 mL of LB medium supplemented with L-tryptophan (100 mg L⁻¹) at 30 °C for 5 days. The supernatants of the cultures were obtained by centrifugation of stationary-phase cultures at 12,000 $\times g$ for 15 min, and the alkaline (pH 8.3) supernatants were adjusted to pH 2.8 with 1 N HCl. Auxins were extracted from the acidified culture medium with equal volumes of ethyl acetate,²⁰ evaporated to dryness, and re-suspended in 1.5 mL of methanol. The samples were passed through 0.2 μ m filters (Orange Scientific GyroDisc CA-PC, Belgium) and analyzed by high-performance liquid chromatography (HPLC, Varian Prostar), with the system equipped with a UV detector and a C-18 column. A mixture of methanol:acetic acid:water (30:1:70, v/v/v) was used as a mobile phase at a rate of 0.6 mL min⁻¹.²¹ Pure IAA was used as a standard. The IAA in the samples was identified and quantified by comparing the retention time and peak area with those of the standard using computer software (Varian Chromatography Systems, Walnut Creek, CA, USA).

Quantification of soluble phosphorus and organic acids in the growth medium

For the estimation of soluble phosphorus, bacterial cultures were grown in three different media, i.e., Pikovskaya medium,²² modified Pikovskaya medium, and LB medium supplemented with tricalcium phosphate (TCP, 5 g L⁻¹). In modified Pikovskaya medium, sucrose was replaced with sodium malate (10 g L⁻¹), which is a preferred C-source for *azospirilla*.¹⁴ The bacterial cultures were grown in conical flasks containing 40 mL of the media at 30 °C for 7 days. Cell-free supernatants were obtained by centrifugation of stationary-phase cultures at 12,000 $\times g$ for 15 min. The supernatants were analyzed to estimate soluble phosphorus by the molybdate blue color method.²³

Organic acids were extracted from cell-free culture media by mixing the supernatant with an equal volume of ethyl acetate. The ethyl acetate phase was separated and evaporated to dryness. Organic acids were re-suspended in 1.5 mL of methanol and filtered through 0.2 μ m filters (Orange Scientific GyroDisc CA-PC, Belgium). The samples were analyzed on a Perkin Elmer (PE) series 200 HPLC system with 20 μ L auto-sampler PE NELSON 900 series interface, a PE NELSON 600 series link, and PE NCI 900 Network Chromatography interface, followed by detection using a diode-array detector at 210 nm and UV spectra (190–400 nm). A Cation H Micro-Guard pre-column, followed by an Aminex HPX-87H analytical column, were used for separation. Sulfuric acid (0.002 mol L⁻¹) was used as a mobile phase with a flow rate of 0.6 mL min⁻¹. Citric acid, malic acid, acetic acid, succinic acid, gluconic acid, lactic acid, and oxalic acid (100 ppm solutions) were used as standards. The peak areas and retention times of the samples were compared with those of the standards for quantification of organic acids produced by the bacterial strains.

Inoculation of wheat plants with *Azospirillum* strains

Three *Azospirillum* strains, AzoK1, AzoK2, and WB3, were used as inoculants for wheat plants (variety Sehar-2006) grown in non-sterile soil pots (0.02 m³). The pots were kept in a net house under natural light and temperature conditions. *Azospirillum* strain WB3 (previously isolated from the rhizosphere of wheat)²⁴; was used as a positive control. The pots were filled with 10 kg of soil (about 27 cm deep) of the Hafizabad series (Aridisol-coarse-loamy, mixed, hyperthermic Ustalfic, Haplargid in the US Department of Agriculture classification). The soil was collected from the top 30 cm of an experimental field of the NIBGE. The pH of this clay loam was 8.3, total N was 0.61 g kg⁻¹, organic matter was 6.2 g kg⁻¹, and available P was 68 mg kg⁻¹. Ten healthy seeds of equal size were sown in five different places at equal distances in each pot. Each treatment was replicated five times. A nitrogen fertilizer at 80% of the recommended dose (1.2 g of urea/pot) was applied to the uninoculated control as well as to all inoculated treatments in two equal split doses, i.e., at the tillering (30 days after sowing) and flowering (75 days after sowing) stages. Before sowing, 80% of the recommended dose of P (0.6 g of diammonium phosphate/pot) was applied to the soil. Bacterial cultures for plant inoculation were grown in 100 mL conical flasks containing 40 mL of LB medium at 30 °C for 24 h. Cell pellets were obtained by centrifugation at 12,000 × *g* for 10 min, and the pellets were re-suspended in 40 mL of sterilized water for use as inocula. One milliliter of inoculum (1 × 10⁸ cells) was added to each plant after 1 week of germination, and 1 mL of sterilized water was applied to the uninoculated controls. The plants were harvested at maturity (180 days after sowing). Plant data (dry weight and grain weight) for 10 plants from each treatment were measured after drying the plant material at 65 °C for 2 days.

Estimation of phosphorus and nitrogen contents in plant material

For estimation of nitrogen contents in shoots and grains, a method described by Van Schouwenberg and Walinge²⁵ was followed. Measurement of phosphorus contents in shoots and grains was carried out by the ammonium vanadate–molybdate method.²⁶

Statistical analysis

Effects of the bacterial isolates on growth parameters of wheat were determined by a completely randomized design analysis of variance (ANOVA) using the Statistix 8.1 software. Mean values and standard errors were also calculated. The means were compared by Fisher's least significant difference test at $\alpha = 0.05$ for all parameters.

Results

Five *Azospirillum*-like strains were isolated from the rhizosphere of wheat. All the isolates formed pinkish colonies on LB agar plates. The cells were rod-shaped and showed spiral motility typical of azospirilla. To further strengthen

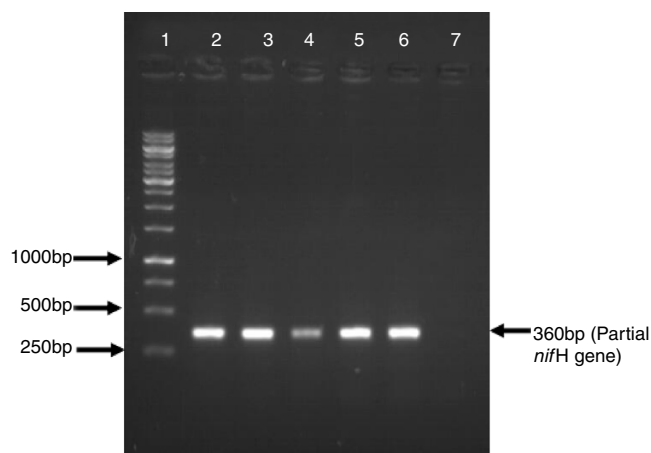


Fig. 1 – PCR-amplification of partial *nifH* from *Azospirillum* strains isolated from wheat rhizosphere. Lane 1: 1 kb ladder (Fermentas, Germany); Lane 2: isolate AzoK1; Lane 3: isolate AzoK2; Lane 4: isolate AzoK3; Lane 5: isolate AzoK4; Lane 6: isolate AzoK5 and Lane 6: negative control.

this observation based on morphological characteristics, a partial sequence of the nitrogenase-encoding gene *nifH* was amplified, which is present in all nitrogen fixers, including azospirilla. A DNA band of the expected size (360 bp) was observed in all strains (Fig. 1).

The BOX-PCR analysis confirmed that the bacterial isolates were different strains and not re-isolates of the same strain. All five strains showed unique banding patterns of the PCR products (Fig. 2). The banding pattern of isolate AzoK1 showed that it was totally different from the other strains. The sizes of the bands were between 250 and 1500 bp. One strong DNA

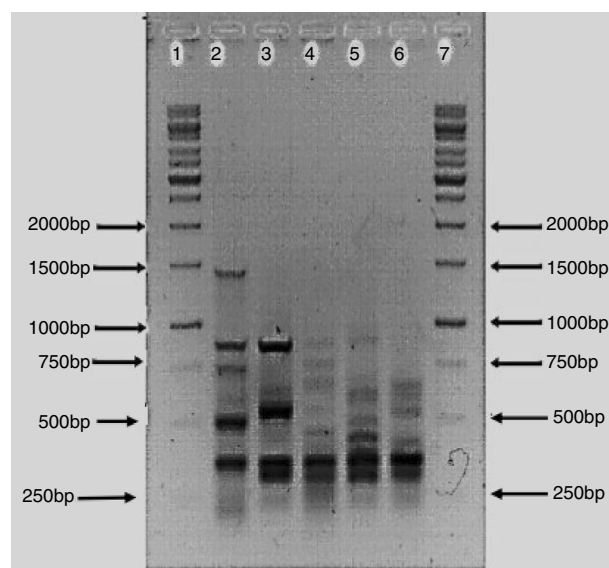


Fig. 2 – Differentiation of *Azospirillum* strains isolated from wheat rhizosphere by BOX-PCR. Lane 1: 1 kb ladder (Fermentas, Germany); Lane 2: isolate AzoK1; Lane 3: isolate AzoK2; Lane 4: isolate AzoK3; Lane 5: isolate AzoK4; Lane 6: isolate AzoK5 and Lane 7: 1 kb ladder (Fermentas, Germany).

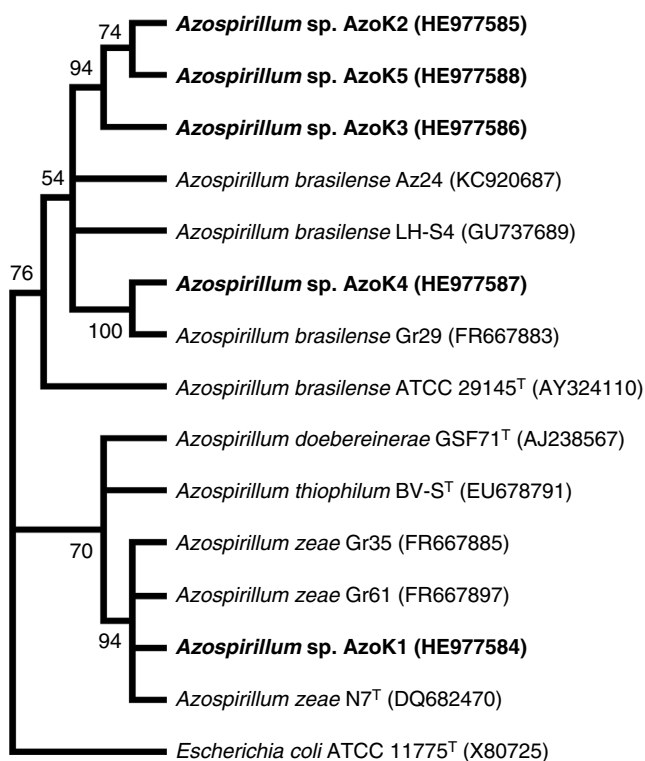


Fig. 3 – Phylogenetic tree based on 16S rRNA gene sequences. The tree was constructed by maximum likelihood method. Bootstrap values over 50% (based on 100 replications) are shown at each node. Accession numbers are given in parentheses. The isolates obtained in the present study are given in bold letters.

band (approximately 300 bp) was present in four strains but absent from AzoK1. Many minor bands of different sizes were also amplified from the four strains.

Azospirillum strain AzoK1 showed maximum 16S rRNA sequence homology (99%) to *Azospirillum zeae*, and the other four isolates shared higher sequence homology with *Azospirillum brasilense* strains. The constructed phylogenetic tree based on the 16S rRNA gene sequences showed that isolate AzoK1 shared a cluster with *A. zeae* (Fig. 3). Three isolates, AzoK2, AzoK3, and AzoK5, formed a cluster with *A. brasilense* strains, and isolate AzoK4 fell in a separate cluster within the main group of *A. brasilense* strains.

IAA production was estimated in LB broth supplemented with L-tryptophan (Table 2). The maximum amount of IAA was produced by *Azospirillum* sp. AzoK2, followed by strains AzoK3 and AzoK4. Only a minor amount of IAA was detected in the culture of *Azospirillum* sp. AzoK1. The quantity of phosphate solubilized by the bacterial strains was determined in three different growth media, i.e., Pikovskaya medium, modified Pikovskaya medium, and TCP-supplemented LB medium. In Pikovskaya medium, phosphate solubilization was not detected for any strain after 7 days of incubation (Table 2). However, phosphate-solubilizing activity of the isolates was detected in modified Pikovskaya medium and TCP-supplemented LB. In modified Pikovskaya medium, the maximum amount of phosphate was solubilized by *Azospirillum* sp. AzoK2. Phosphate solubilization was not detected in modified Pikovskaya medium for *Azospirillum* sp. WB3 and AzoK4. All the *Azospirillum* strains solubilized phosphate in TCP-supplemented LB, except *Azospirillum* sp. AzoK4 (Table 2). The maximum amount of phosphate was solubilized by *Azospirillum* sp. AzoK2.

Organic acids were detected in cell-free supernatants of modified Pikovskaya medium and TCP-supplemented LB after 7 days of incubation. Acetic, citric, lactic, malic, and succinic acids were detected in almost all *Azospirillum* cultures. In modified Pikovskaya medium, acetic and malic acids were detected in higher amounts in the cell-free supernatants of all *Azospirillum* strains, but relatively minor amounts of lactic acid were present in this medium (Fig. 4A). In TCP-supplemented LB medium, acetic acid was produced by all strains in significant amounts (Fig. 4B). The maximum amounts of citric acid were produced by three isolates, except AzoK1 and AzoK4, in this medium. A significant amount of succinic acid was only produced by AzoK2, and malic acid was one of the major acids detected in the supernatants of two strains, WB3 and AzoK5.

The maximum plant shoot dry weight (8.95 ± 0.20 g) and grain weight (6.17 ± 0.11 g) were recorded in the strain AzoK1-inoculated treatment, and the increases in the plant shoot and grain weights over the uninoculated control were 19 and 15%, respectively (Table 3). The nitrogen and phosphorus contents in the grains and shoots of the inoculated plants were not significantly different from those of the control plants (Table 4).

The maximum plant shoot dry weight (8.95 ± 0.20 g) and grain weight (6.17 ± 0.11 g) were recorded in the strain AzoK1-inoculated treatment, and the increases in the plant shoot and grain weights over the uninoculated control were 19 and 15%, respectively (Table 3). The nitrogen and phosphorus contents in the grains and shoots of the inoculated plants were not significantly different from those of the control plants (Table 4).

Discussion

In the present study, the rhizosphere soil of field-grown wheat plants was collected and used for isolation of *Azospirillum*-like strains, which are known to improve the growth and productivity of many agricultural crop species.²⁷ *Azospirillum* have been isolated from roots of numerous wild and cultivated grasses, cereals, and legumes and from tropical, subtropical, and temperate soils.²⁸ In the N-free growth medium, which is selective for *Azospirillum*,¹⁴ all the isolates showed excellent growth and characteristic spiral motility. On LB agar plates, they formed pinkish, wrinkled colonies. Furthermore, a partial *nifH* sequence was successfully amplified from all the isolates, indicating that they are potential nitrogen fixers, similar to *azospirilla*. The cell morphology and colony color of all the *Azospirillum*-like isolates obtained in the present study were similar.

BOX-PCR polymorphism patterns have been effectively used for differentiation of bacterial strains.²⁹ All the *Azospirillum* isolates included in this study exhibited unique, strain-specific banding patterns obtained by BOX-PCR. *Azospirillum* strain AzoK1 showed a BOX-PCR banding pattern significantly different from those of the other strains. One DNA band of about 300 bp was amplified from four strains but was absent from the *Azospirillum* strain AzoK1 profile, indicating that the latter strain belongs to a different group or species.

On the basis of the sequence analysis of the 16S rRNA gene, isolates AzoK2, AzoK3, AzoK4, and AzoK5 were 96–99% similar to *A. brasilense* strains, and the fifth strain, AzoK1, showed high sequence homology (99%) with *A. zeae*. Isolate AzoK1 shared a

Table 2 – Phosphate solubilization and indole acetic acid (IAA) production by *Azospirillum* strains (Mean $\pm$ SD).

Name of the isolates	Indole acetic acid (mgL ⁻¹) with tryptophan	Solubilized P (mgL ⁻¹) in Pikovskaya medium	Solubilized P (mgL ⁻¹) in modified Pikovskaya medium	Solubilized P (mgL ⁻¹) in TCP-supplemented LB medium
<i>Azospirillum</i> sp. AzoK1	0.84 $\pm$ 0.75	ND	52.40 $\pm$ 3.42	62.10 $\pm$ 1.65
<i>Azospirillum</i> sp. AzoK2	30.49 $\pm$ 1.04	ND	72.00 $\pm$ 2.94	105.50 $\pm$ 4.93
<i>Azospirillum</i> sp. AzoK3	24.35 $\pm$ 0.91	ND	26.55 $\pm$ 2.01	44.60 $\pm$ 2.91
<i>Azospirillum</i> sp. AzoK4	24.33 $\pm$ 3.03	ND	ND	ND
<i>Azospirillum</i> sp. AzoK5	19.15 $\pm$ 1.34	ND	39.45 $\pm$ 3.24	60.50 $\pm$ 2.16
<i>Azospirillum</i> sp. WB3	14.15 $\pm$ 1.48	ND	ND	43.65 $\pm$ 2.15

ND, not detected; SD, standard deviation.

cluster with the *A. zeae* type strain, and the other four isolates formed a cluster with *A. brasilense* strains.

Our findings revealed that the wheat rhizosphere is colonized by *A. brasilense*- as well as *A. zeae*-related isolates. This is the first report indicating the presence of an *A. zeae*-like isolate in the wheat rhizosphere in Pakistan. The *A. zeae* type strain was originally isolated from roots of maize in Canada.³⁰ Originally, this isolate was identified and reported as *Azospirillum*

*lipoferum*³¹ but was later re-identified on the basis of a polyphasic taxonomic approach, including morphological characterization, Biolog analysis, DNA–DNA hybridization, and 16S rRNA, *cpn60*, and *nifH* sequence analysis.³⁰ Venieraki et al.³² isolated three *Azospirillum* strains from the rhizosphere of wheat grown in Greece and showed that the isolates recovered were *A. zeae* by phylogenetic analysis based on 16S rRNA gene sequences.

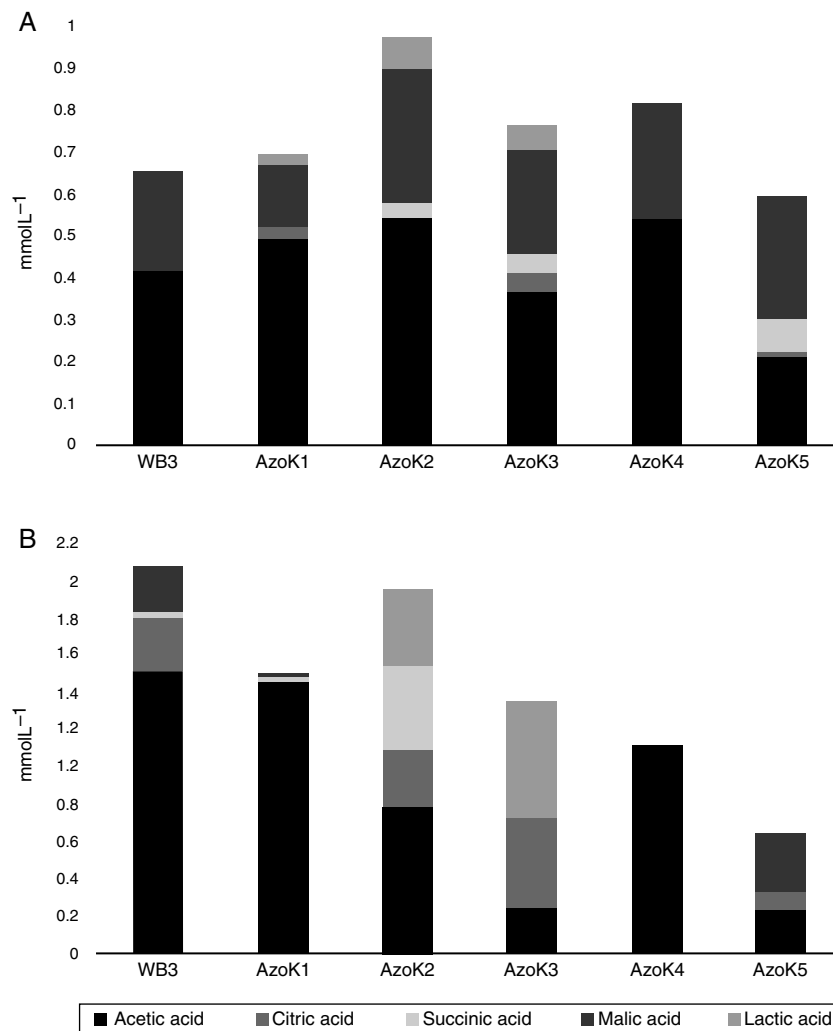


Fig. 4 – Production of organic acids by bacterial isolates grown in modified Pikovskaya medium (A) and TCP-supplemented LB (B). *Azospirillum* strains WB3, AzoK1, AzoK2, AzoK3, AzoK4 and AzoK5 were used.

Table 3 – Effect of bacterial inoculation on growth of wheat plants in the soil pot experiment.

S. No.	Name of treatments	Shoot dry weight (g plant ⁻¹) ^a	Grain weight (g plant ⁻¹) ^a
1	<i>Azospirillum</i> sp. AzoK1	8.95 A	6.17 A
2	<i>Azospirillum</i> sp. AzoK2	8.89 A	6.02 B
3	<i>Azospirillum</i> sp. WB3	8.22 B	5.97 B
4	Non-inoculated control	7.52 C	5.35 C
	LSD (5%)	0.37	0.13

LSD, least significant difference.
^a Ten plants from each treatment were used for the analysis.

All the *Azospirillum* strains tested in the present study failed to grow and solubilize P in the commonly used Pikovskaya medium. It has been reported that azospirilla cannot utilize disaccharides, such as sucrose, which is added to Pikovskaya medium as a sole C-source.^{33,34} As a result, the *Azospirillum* strains did not solubilize hardly soluble phosphorus, such as TCP. *Azospirillum* use organic acids, such as malic, succinic, α-ketoglutaric, gluconic, and lactic acids, as their preferred carbon sources.³³ Therefore, Pikovskaya medium was modified by replacing sucrose with Na-malate and used to study P solubilization by the *Azospirillum* strains. The tested *Azospirillum* strains produced organic acids in the growth medium. Acetic acid was produced by all strains, including AzoK4, but the latter showed no P-solubilizing activity. A very small amount of malic acid was detected in all culture supernatants compared to the control, showing that most of the malate added to the modified Pikovskaya medium was consumed by the bacteria during incubation.

Phosphate-solubilizing activity of the *Azospirillum* strains was also studied in TCP-supplemented LB medium. All the strains solubilized phosphate, except *Azospirillum* sp. AzoK4. The amount of soluble phosphate, determined in the TCP-supplemented LB medium, was higher compared to the modified Pikovskaya medium. *Azospirillum* sp. AzoK2 produced the maximum amount of soluble phosphorus, followed by *Azospirillum* sp. AzoK1. *Azospirillum* sp. AzoK4 failed to show phosphate solubilization and produced only acetic acid in the TCP-supplemented LB medium. In this growth medium, organic acids, e.g., acetic, citric, malic, lactic, and succinic acids, were produced by the other strains tested in the present study, but gluconic and α-ketoglutaric acids were not detected. Goebel and Krieg³⁵ showed that gluconic acid was not formed during growth of *A. lipoferum* and *A. brasilense* strains on fructose and was only detected in an *A. lipoferum*

culture during growth on glucose. In the same study, it was reported that *A. brasilense* showed only slight uptake of glucose and extremely slow growth on this carbon source. Rodriguez et al.¹⁰ reported that *Azospirillum* strains could produce gluconic acid *in vitro* when grown on fructose amended with glucose as an inducer of gluconic acid production and also showed *in vitro* phosphate-solubilizing capabilities. This has suggested that phosphate solubilization by these strains is mediated by glucose or gluconic acid metabolism. Since solubilization of phosphate preceded the detection of gluconic acid in the medium, it is possible that even low levels of the acid (below the HPLC detection limit) started to dissolve sparingly soluble phosphate. Alternatively, consumption of gluconic acid by growing cells could have taken place. Furthermore, it has been reported that *Azospirillum halopraeferens*, a bacterium that does not use glucose and consequently does not produce acid, can solubilize insoluble inorganic phosphate *in vitro* by an unknown mechanism.³⁶

Bacterial isolates obtained from the rhizosphere of various plants have been shown to produce IAA in pure culture.³⁷ In the present study, all the bacterial strains produced significant amounts of IAA in the culture medium, except *Azospirillum* sp. AzoK1. Under natural conditions, plant roots excrete organic compounds, including L-tryptophan, which can be utilized by rhizobacteria for IAA biosynthesis.⁶ Perrig et al.³⁸ reported IAA production ranging from 2.9 to 10.8 mg L⁻¹ by two agronomically important *Azospirillum* strains. Fatima et al.³⁹ also detected IAA in the range from 19.4 to 30.2 mg L⁻¹ in *A. brasilense* cultures. Venieraki et al.⁴⁰ isolated two *A. zeae* strains from the rhizosphere of wheat and found that both produced IAA (29.8 and 194.8 mg L⁻¹, respectively). In the present study, the amount of IAA produced by *Azospirillum* sp. AzoK1 in tryptophan-amended LB was 0.84 mg L⁻¹, which is very low compared to previous reports. The higher levels of IAA

Table 4 – Effect of *Azospirillum* inoculation on the nitrogen and phosphorus contents of wheat plants (shoot and grain) in the soil pot experiment (mean ± SD).

Treatments	Nitrogen contents (g kg ⁻¹) ^a		Phosphorus contents (g kg ⁻¹) ^a	
	Shoot	Grain	Shoot	Grain
1. <i>Azospirillum</i> sp. AzoK1	4.46 ± 0.40	23.33 ± 1.00	0.59 ± 0.02	3.16 ± 0.20
2. <i>Azospirillum</i> sp. AzoK2	4.45 ± 0.30	23.34 ± 1.00	0.60 ± 0.01	3.17 ± 0.07
3. <i>Azospirillum</i> sp. WB3	4.40 ± 0.70	23.30 ± 1.50	0.59 ± 0.01	3.16 ± 0.04
4. Non-inoculated control	4.44 ± 0.50	23.31 ± 1.00	0.59 ± 0.03	3.16 ± 0.03

SD, standard deviation.
^a Ten plants from each treatment were used for the analysis.

production reported by Venieraki et al.⁴⁰ could be due to a different IAA detection method (spectrophotometry rather than HPLC) or to the fact that a different growth medium (NFB) was used for bacterial growth. *Azospirillum* is a rhizospheric bacterium that has been reported to increase the yield of wheat plants by producing phytohormones and growth regulators and by providing salt stress relief.⁷ Based on the studies on maize and wheat, Hungria et al.⁴¹ proposed that the use of inoculants containing *Azospirillum* might help reach the goal of reducing the use of chemical fertilizers globally. In the present study, all the inoculated strains showed a positive effect on plant growth compared to the uninoculated control. The maximum increases in shoot dry weight (19%) and grain weight (15%) over the uninoculated control were recorded for the plants inoculated with *Azospirillum* sp. Azok1. The nitrogen and phosphorus contents of the grains and shoots of the inoculated plants were not significantly different from those of the control plants, suggesting that the growth promotion might have been mainly due to phytohormone production by the inoculated strains. Prinsen et al.⁴² reported that inoculation of wheat with *Azospirillum* resulted in an increase of seed yield by 7.4–10.31% in field trials where no N fertilization had been applied. Díaz-Zorita and Fernández-Canigia⁴³ demonstrated that a liquid formulation of *A. brasilense* increased the number of harvested grains by 6.1% and the grain yield of wheat by 260 kg ha⁻¹ (8.0%). Inoculation with *Azospirillum* has been found to affect early plant and root development, shoot and root dry weight, grain yield, and the nitrogen uptake efficiency of plants.⁴⁴

In the present study, all the isolates exhibited plant growth-promoting traits in pure culture and also improved plant growth in pot experiments. Therefore, these strains qualify as potential candidates to be used for production of biofertilizers in Pakistan and should be further evaluated as inoculants in field trials.

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Babalola OO. Beneficial bacteria of agricultural importance. *Biotechnol Lett.* 2010;32(11):1559–1570.
2. Helman Y, Burdman S, Okon Y. Plant growth promotion by rhizosphere bacteria through direct effects. In: Rosenberg E, Gophna U, eds. *Beneficial Microorganisms in Multicellular Life Forms*. Heidelberg, Germany: Springer; 2011:89–103.
3. Spaepen S, Vanderleyden J, Okon Y. Plant growth-promoting actions of rhizobacteria. In: Loon LCV, ed. *Advances in Botanical Research*. vol. 51. Amsterdam, The Netherlands: Academic Press, Elsevier; 2009:283–320 [chapter 7].
4. Bashan Y, Holguin G. *Azospirillum*-plant relationships: environmental and physiological advances (1990–1996). *Can J Microbiol.* 1997;43(2):103–121.
5. Malhotra M, Srivastava S. Stress-responsive indole-3-acetic acid biosynthesis by *Azospirillum brasilense* SM and its ability to modulate plant growth. *Eur J Soil Biol.* 2009;1(45):73–80.
6. Hayat R, Ali S, Amara U, Khalid R, Ahmed I. Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann Microbiol.* 2010;60(4):579–598.
7. Arzanesh M, Alikhani H, Khavazi K, Rahimian H, Miransari M. Wheat (*Triticum aestivum* L.) growth enhancement by *Azospirillum* sp. under drought stress. *World J Microbiol Biotechnol.* 2011;27(2):197–205.
8. Alamri SA, Mostafa YS. Effect of nitrogen supply and *Azospirillum brasilense* Sp-248 on the response of wheat to seawater irrigation. *Saudi J Biol Sci.* 2009;16(2):101–107.
9. Khade SW, Rodrigues BF, Sharma PK. Arbuscular mycorrhizal status and root phosphatase activities in vegetative *Carica papaya* L. varieties. *Acta Physiol Plant.* 2010;32(3):565–574.
10. Rodriguez H, Gonzalez T, Goire I, Bashan Y. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. *Naturwissenschaften.* 2004;91(11):552–555.
11. Tahir M, Mirza MS, Zaheer A, Dimitrov MR, Smidt H, Hameed S. Isolation and identification of phosphate solubilizer *Azospirillum*, *Bacillus* and *Enterobacter* strains by 16S rRNA sequence analysis and their effect on growth of wheat (*Triticum aestivum* L.). *Aust J Crop Sci.* 2013;7(9):1284–1292.
12. Vazquez P, Holguin G, Puente M, Lopez-Cortes A, Bashan Y. Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biol Fertil Soils.* 2000;30(5–6):460–468.
13. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil.* 2003;255:571–1157.
14. Okon Y, Albrecht SL, Burris R. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. *Appl Environ Microbiol.* 1977;33(1):85–88.
15. Maniatis T, Fritsch EF, Sambrook J. *Molecular Cloning: A Laboratory Manual*. vol. 545. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1982:200–201.
16. Wilson K. Preparation of genomic DNA from bacteria. In: Ausubel FM, ed. *Current Protocols in Molecular Biology*. New York, United States: John Wiley & Sons, Inc.; 2001 [unit 2.4].
17. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 1997;25(24):4876–4882.
18. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, ed. *Mammalian Protein Metabolism*. vol. 3. New York: Academic Press; 1969:21–132.
19. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol.* 2013;30(12):2725–2729.
20. Tien T, Gaskins M, Hubbell D. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl Environ Microbiol.* 1979;37(5):1016–1024.
21. Rasul G, Mirza MS, Latif F, Malik KA. Identification of plant growth hormones produced by bacterial isolates from rice, wheat and kallar grass. In: Malik KA, Mirza MS, Ladha JK, eds. *Nitrogen Fixation With Non-Legumes*. vol. 79. Netherlands: Springer; 1998:25–37.
22. Pikovskaya R. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya.* 1948;17:362–370.
23. Watanabe F, Olsen S. Test of an ascorbic acid method for determining phosphorus in water and NaHCO₃ extracts from soil. *Soil Sci Soc Am J.* 1965;29(6):677–678.
24. Malik KA, Mirza MS, Hassan U, et al. The role of plant-associated beneficial bacteria in rice-wheat cropping system. In: Kennedy IR, Choudhury ATMA, eds. *Biofertilisers in Action. A Report for the Rural Industries Research and Development Corporation, Canberra.* 2002:73–83.

25. Van Schouwenberg J, Walinge I. *Methods of Analysis for Plant Material*. Wageningen, The Netherlands: Agricultural University; 1973.
26. Chapman HD, Pratt PF. Ammonium vanadate–molybdate method for determination of phosphorus. In: Chapman HD, Pratt PF, eds. *Methods of Analysis for Soils, Plants and Water*. vol. 1. Berkeley, USA: University of California; 1961:184–203.
27. Okon Y, Labandera-Gonzalez CA. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. *Soil Biol Biochem*. 1994;26(12):1591–1601.
28. Saikia SP, Bora D, Goswami A, Mudoi KD, Gogoi A. A review on the role of *Azospirillum* in the yield improvement of non leguminous crops. *Afr J Microbiol Res*. 2012;6(6):1085–1102.
29. Louws FJ, Fulbright DW, Stephens CT, De Bruijn F. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl Environ Microbiol*. 1994;60(7):2286–2295.
30. Mehnaz S, Weselowski B, Lazarovits G. *Azospirillum zeae* sp. nov., a diazotrophic bacterium isolated from rhizosphere soil of *Zea mays*. *Int J Syst Evol Microbiol*. 2007;57(12):2805–2809.
31. Mehnaz S, Lazarovits G. Inoculation effects of *Pseudomonas putida*, *Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. *Microb Ecol*. 2006;51(3):326–335.
32. Venieraki A, Dimou M, Pergalis P, Kefalogianni I, Chatzipavlidis I, Katinakis P. The genetic diversity of culturable nitrogen-fixing bacteria in the rhizosphere of wheat. *Microb Ecol*. 2011;61(2):277–285.
33. Westby C, Cutshall D, Vigil G. Metabolism of various carbon sources by *Azospirillum brasilense*. *J Bacteriol*. 1983;156(3):1369–1372.
34. Mehnaz S, Weselowski B, Lazarovits G. *Azospirillum canadense* sp. nov., a nitrogen-fixing bacterium isolated from corn rhizosphere. *Int J Syst Evol Microbiol*. 2007;57(3):620–624.
35. Goebel EM, Krieg NR. Fructose catabolism in *Azospirillum brasilense* and *Azospirillum lipoferum*. *J Bacteriol*. 1984;159(1):86–92.
36. Seshadri S, Muthukumarasamy R, Lakshminarasimhan C, Ignacimuthu S. Solubilization of inorganic phosphates by *Azospirillum halopraeferens*. *Curr Sci India*. 2000;79(5):565–567.
37. Flores K, Haderl K. The random walk of *Azospirillum brasilense*. *J Biol Dyn*. 2010;4(1):71–85.
38. Perrig D, Boiero M, Masciarelli O, et al. Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. *Appl Microbiol Biotechnol*. 2007;75(5):1143–1150.
39. Fatima Z, Saleemi M, Zia M, et al. Antifungal activity of plant growth-promoting rhizobacteria isolates against *Rhizoctonia solani* in wheat. *Afr J Biotechnol*. 2009;8(2):219–225.
40. Venieraki A, Dimou M, Vezyri E, et al. Characterization of nitrogen-fixing bacteria isolated from field-grown barley, oat, and wheat. *J Microbiol*. 2011;49(4):525–534.
41. Hungria M, Campo RJ, Souza EM, Pedrosa FO. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant Soil*. 2010;331(1–2):413–425.
42. Prinsen E, Costacurta A, Michiels K, Vanderleyden J, Van Onckelen H. *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a non-tryptophan dependent pathway. *Mol Plant Microbe Interact*. 1993;6:609.
43. Díaz-Zorita M, Fernández-Canigia MV. Field performance of a liquid formulation of *Azospirillum brasilense* on dryland wheat productivity. *Eur J Soil Biol*. 2009;45(1):3–11.
44. Dobbelaere S, Vanderleyden J, Okon Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit Rev Plant Sci*. 2003;22(2):107–149.
45. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res*. 1989;17(19):7843–7853.
46. Poly F, Monrozier LJ, Bally R. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol*. 2001;152(1):95–103.